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The comparisons in protective mechanisms and efficiencies among dietary α-lipoic acid, β-glucan and L-carnitine on Nile tilapia infected by *Aeromonas hydrophila*

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ABSTRACT

Dietary α-lipoic acid (LA), β-glucan (Gluc) and L-carnitine (L-Ca) are commonly used additives to promote fish growth and stress resistance in aquaculture production. However their mechanisms and efficiencies in helping fish to resist diseases have not been compared before. In this study, we fed Nile tilapia (*Oreochromis niloticus*) with diets containing appropriate doses of LA, Gluc and L-Ca for five weeks and further intraperitoneally injected the fish with *Aeromonas hydrophila*. After dietary treatment, none of the additives affected the fish growth, but dietary Gluc and L-Ca reduced protein and lipid body contents in fish, respectively. After *A. hydrophila* challenge, all fish treated with the three dietary additives showed higher survival rate, but those fed on dietary L-Ca had lower survival than those fed on LA and Gluc diets, indicating high protection efficiency of LA and Gluc. The protective mechanisms of the three feed additives were quite different under *A. hydrophila* infection. Dietary LA induced higher total antioxidant capacity and higher mRNA expression of anti-oxidative genes than other additives in liver and also activated partly the immune function in serum and spleen. Gluc largely increased the immune function by activating the immunity enzymes in serum, inducing inflammation in liver and increasing the expression of immune genes in spleen and head kidney. Gluc also increased partly the antioxidant capacity in serum and liver and lipid catabolism in liver. L-Ca largely increased lipid catabolism in liver while it increased partly the antioxidant capacities in serum and liver. Taken together, these results indicate that, dietary LA, Gluc and L-Ca have various protective mechanisms and differ in their efficiencies on resisting *A. hydrophila* infection in Nile tilapia.

1. Introduction

Aquaculture continues to play an important role in food production in the world by providing high quality protein to people every year [1]. However, aquaculture industry is increasingly threatened by various infectious diseases which cause huge economic loss in production and pose risks to consumer health [2]. Therefore, identifying mechanisms and prevention methods to resist pathogens infections are urgent issues required for sustainable aquaculture production. It is generally known that innate immunity and adaptive immunity are the two defense immune systems used by fish to resist bacterial infections [3]. Apart from the immune system, reduction in oxidative stress, which damages lipids, proteins and DNA and causes cell damages and apoptosis, also protects animals from bacterial infections [4–6]. Energy metabolism is another important process in resisting infections because immunity reactions are energy-consuming processes and the immune function is closely correlated to energy metabolism in mammals [7,8]. Indeed, decreasing lipid catabolism in Nile tilapia (*Oreochromis niloticus*) by inhibiting mitochondrial β-oxidation decreased the resistance of fish to bacterial infection [9].

Dietary additives are widely used in aquaculture to promote growth performance and many of them help fish to resist bacterial infections. Alpha-lipoic acid (LA), β-glucan (Gluc) and L-carnitine (L-Ca) are the typical additives which act as antioxidant, immunostimulant and metabolic regulator, respectively. Dietary LA is a common used antioxidant because it is easily metabolized into its reduced form, dihydrolipoic acid, which accumulates in various tissues. In fish, LA reduces oxidative stress by increasing glutathione concentration or antioxidant

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enzymes activities, decreasing lipid peroxidation and reactive oxygen species (ROS) concentration and modulating genes expression involved in antioxidant mechanism [10–12]. Accordingly, LA has been shown to protect aquatic animals against oxidative stress induced by microcystin toxicity [13] and environmental stresses [14]. Recently, LA has also been found to induce immune function in grass carp (*Ctenopharyngodon idella*) [15]. Dietary β-glucan is a widely-accepted immunostimulant, which is able to enhance the immune response and prevent disease outbreaks [2] because of its ability to be recognized by various immunity receptors such as the toll-like receptors (TLR). The capacity of β-glucan to protect fish from pathogen infections by increasing the innate immunity have been well established in various fish species, including Nile tilapia [16,17], zebrafish (*Danio rerio*) [18], rainbow trout (*Oncorhynchus mykiss*) [19] and common carp (*Cyprinus carpio*) [20]. Moreover, β-glucan has also been found to activate the innate immunity and antioxidant enzymes activities [21,22]. Dietary L-Ca is used to promote lipid utilization and regulate energy metabolism in many fish species [23]. It is an essential compound in transporting long-chain fatty acids into mitochondria for β-oxidation and also promotes the release of coenzyme A to facilitate β-oxidation and tricarboxylic acid cycle (TAC) pathways [24]. The addition of L-Ca in diets promotes the utilization of body lipid and conserves dietary protein in fishes such as channel catfish (*Ietalurus Punetaus*) [25], silver perch (*Bidyanus bidyanus*) [26] and Nile tilapia [27]. Furthermore, L-Ca has been found to enhance the resistance of bacterial infection in turbot (*Scophthalmus maximus*) [28] and black carp (*Mylopharyngodon piceus*) by increasing immune function [29].

It is clear from the above studies that, dietary LA, Gluc and L-Ca have different properties and function targets. However, it is also interesting to note that, they all have a common phenotype of protecting fish from diseases and stresses. Unlike Gluc, information on the ability of LA and L-Ca to modulate immunity in fish is currently limited. Presently, it is unknown whether LA and L-Ca could induce the immune responses of fish as efficiency as Gluc because previous works studied them separately and no any attempt was done to compare their mechanisms in resisting infectious diseases. Furthermore, information on the efficiencies of dietary LA, Gluc and L-Ca is unavailable, which hinders the application of these additives in aquaculture production. Moreover, it is also not clear whether LA and L-Ca help fish to resist disease by only reducing oxidative stress and improving energy supply, respectively.

Nile tilapia is one of the most important aquaculture species around the world. However, its culture is threatened by bacteria diseases such as bacterial septicemia caused by *A. hydrophila* infection, which causes high mortality in its production [30]. Luckily, the optimum concentrations of LA, Gluc and L-Ca have been well established in Nile tilapia [17,31,32]. Therefore, we selected Nile tilapia to compare the differences in protective mechanisms and efficiencies of the three dietary additives at optimal doses. Nile tilapia were pretreated with diets containing LA, Gluc or L-Ca for five weeks, subsequently were infected by injecting them with *A. hydrophila* to compare their protective functions. The results obtained demonstrate the ability of all the three additives to help Nile tilapia to resist *A. hydrophila* infection. However, the three dietary additives use diverse mechanisms including immunity stimulation, antioxidants activation and energy metabolism to protect fish from bacterial infections. They also have differences in resistance efficiencies, with L-Ca depicting lower capacity than that of LA and Gluc diets. For the first time, our study compared the differences in protective mechanisms and efficiencies of dietary additives in resisting disease in fish under their optimal doses, which provides important information for their use in aquaculture.

2. Materials and methods

2.1. Animal ethics

All experiments were conducted strictly under the Guidance of the Care and Use of Laboratory Animals in China. This study was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.2. Experimental fish and culture conditions

About 500 juvenile Nile tilapia were obtained from Aquaculture Genetics and Breeding Technology center at Shanghai Ocean University (Shanghai, China). Before the formal experiment, fish were maintained in three 200-L tanks (each about 170 fish) at 27 ± 1 °C for three weeks. During this period, the fish were hand-fed twice daily by using a commercial diet (Chengdu, China) containing 33% protein and 5% lipid.

2.3. Diets preparation

Balanced basal diets with or without additives were prepared (Table 1). The three experimental diets were basal diet supplemented with α-lipoic acid (600 mg/kg, Aladdin, China), β-glucan (70% effective, 1.5 g/kg, Aladdin, China) and L-carnitine (12.5 g/kg, Aladdin, China), hereafter referred to as LA, Gluc and L-Ca, respectively. The doses of α-lipoic acid was obtained from the studies in hybrid tilapia [31] and grass carp [33]. The dose of β-glucan was referred to the studies in Nile tilapia [17], rainbow trout [19] and pompano fish [34]. The dose of L-carnitine has been applied in Nile tilapia [32] and we have used it our previous study in zebrafish [35]. The macro and micro-ingredients were mixed together first, then oil and de-chlorinated water were add into the ingredients and mixed. The α-lipoic acid was dissolved in oil, while β-glucan and L-carnitine were dissolved in water

Table 1
Formulation of the experimental diets.

Feed composition (g/kg)	Control	α-Lipoic acid	β-Glucan	L-Carnitine
Casein	320	320	320	320
Gelatin	80	80	80	80
Mixed oil[a]	70	70	70	70
Corn starch	300	300	300	300
Mixed vitamin[b]	10	10	10	10
Mixed minerals[c]	15	15	15	15
Carboxy methyl cellulose (CMC)	30	30	30	30
Cellulose	157.75	157.15	154.75	145.25
Choline chloride	5	5	5	5
Butylated hydroxytoluene (BHT)	0.25	0.25	0.25	0.25
$Ca(H_2PO_4)_2$	12	12	12	12
α-Lipoic acid	0	0.6	0	0
β-Glucan	0	0	1.5	0
L-Carnitine	0	0	0	12.5
Total quantity	1000	1000	1000	1000
Proximate composition (g/kg)				
Total protein	316.5	316.5	316.5	316.5
Total lipid	70	70	70	70
Total carbohydrate	300	300	300	300

[a] Fish oil and Soybean oil 1:1.

[b] Mixed vitamin (mg or IU/kg): 500,000 I.U. Vitamin A, 50,000 I.U. Vitamin D3, 2500 mg Vitamin E, 1000 mg Vitamin K3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 mg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Cholin, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C.

[c] Mixed minerals (g/kg): 147.4 g $MgSO_4 \cdot 7H_2O$; 49.8 g NaCl; 10.9 g Fe(II) gluconate; 3.12 g $MnSO_4 \cdot H_2O$; $ZnSO_4 \cdot 7H_2O$; 0.62 g $CuSO_4 \cdot 5H_2O$; 0.16 g kJ; 0.08 g $CoCl_2 \cdot 6H_2O$; 0.06 g NH_4 molybdate; 0.02 g $NaSeO_3$.

before they were mixed with other dietary components. Diets were extruded into 2 mm pellets, air-dried and then stored at −20 °C until needed for use.

2.4. Dietary experiment design and sampling techniques

After acclimation, 240 fish with relatively similar initial weights (2.0 ± 0.1 g) were selected and divided into four groups (three replicates per group, 20 fish per replicate) and fed with control, LA, Gluc and L-Ca diets. During dietary treatment, fish were hand-fed twice daily (9:00 a.m. and 17:00 p.m.) at a feeding rate of 4% body weight per day, under a 14 h light/10 h dark cycle at 26–28 °C. The weight of fish was recorded every 12 days and the amount of feed per group was adjusted accordingly. After five weeks, all fish in each group were counted for survival rate and weighed for weight gain (WG) estimation. Afterwards, nine fish from each group (three per tank) were sampled and sacrificed individually by anesthetizing using MS-222 (20 mg/L) (tricaine methanesulfonate, Western Chemicals, Inc., Ferndale, Washington) for measurement of body indices. The WG, survival rate, hepatosomatic index (HSI), viscerosomatic index (VSI) and visceral adipose index (VAI) measurements were calculated using the following formulae:

Weight gain (WG, %) = 100 × (Final fish weight − Initial fish weight) / Initial fish weight

Survival (%) = 100 × (Final fish number / Initial fish number)

Hepatosomatic index (HSI, %) = 100 × (Liver weight / Body weight)

Viscerosomatic index (VSI, %) = 100 × (Visceral weight / Body weight)

Visceral adipose index (VAI, %) = 100 × (Visceral adipose weight / Body weight)

2.5. Fish body composition analysis

Another nine fish per group (three per tank) were sacrificed individually as described before for body composition analysis using standard methods [36]. The total protein of whole fish body was determined by a semi-automatic Kjeldahl System (FOSS, Sweden) after acid digestion. The total lipid content of whole fish was determined by the Soxhlet method. The ash content was measured by first carbonizing completely the samples on a heating plate (SuDa, China) and then incinerated in a muffle furnace (Peaks, Japan) at 550 °C for 5 h. Nitrogen free extract was calculated by subtracting the sum of moisture, protein, lipid and ash from 100.

2.6. A. hydrophila experimental preparation, injection and sampling

The *A. hydrophila* was purchased from China General Microbiological Culture Collection Center and prepared by culturing them in a Luria Broth (LB) at 37 °C for 12 h with constant shaking (250 rpm). Then cells were harvested by centrifugation (3000 rpm, 10 min), washed and re-suspended in phosphate buffer saline (PBS) (pH 7.4). The bacterial count was determined by standard dilution and plating methods, and then the bacterial fluid were diluted to the density of 2×10^8 CFU/ml. The mean lethal dose (LD_{50}) values were calculated before final injection challenge, and the 14d LD_{50} (3×10^6 CFU/g) were used to inject fish from the four groups.

After sampling fish for growth and body composition, twenty five of the remaining Nile tilapia from each group were intraperitoneally injected with *A. hydrophila*. During this period, fish were hand-fed once a day at 1% of the body weight and feeding was stopped when they started dying. The water in each tank was not replaced. After 5 days of infection (when the infection symptoms were visible in all groups), nine fish per group were euthanized by MS-222 (tricaine methanesulfonate, 20 mg/L) and their individual blood, liver, spleen and head kidney were collected for biochemical analysis. Blood samples were withdrawn from the caudal peduncle by using 1 mL syringe with 21 gauge needles. The blood from two or three fish were mixed to ensure enough samples. Blood samples were immediately centrifuged at 3000 rpm for 10 min at 4 °C, the serum was placed into polypropylene tubes. The remaining fish after sampling were monitored continuously for survival rate and the mortality was recorded daily for 18 days. The bacteria challenge test was repeated using similar experimental conditions in order to confirm the results. During both bacteria challenges, a fish was considered dead when remained motionless and the gill operculum stopped moving. The dead fish from each tank when found were removed.

2.7. Biochemical parameters analysis

The activities of lysozyme, acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), catalase (CAT), the total antioxidant capacity (T-AOC) and malondialdehyde (MDA) concentration of the sampled tissues were measured using commercial kits (Jiancheng Biotech Co., China). All measurement steps were performed according to the relevant kit protocol.

2.8. Total RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis

Total RNA of tissues were isolated by using a Tri Pure Reagent (Aidlab, China) according to the manufacturer's protocol. The quality and quantity of total RNA were tested by NANODROP 2000 Spectrophotometer (Thermo, USA). The cDNAs were synthesized using a PrimerScriptTM RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Takara, Japan) according to the manufacturer's protocol. The qRT-PCR was run in a CFX Connect Real-Time System (Bio-Rad, USA). The conditions for qRT-PCR were 95 °C for 10 min, 40 cycles of 95 °C for 5 s and 60 °C for 18 s. The qRT-PCR reaction was performed in a 20 μl mixture contained 2 μl diluted cDNA, 10 μl 2 × Ultra SYBR Mixture (Aidlab, China), 2 μl forward and reverse each gene-specific primer and 6 μl distilled water. Melting curves of amplified products were generated to ensure the specificity of assays at the end of each qRT-PCR. All primers used in this work were specific and the amplification efficiency of primers were between 98% and 102%. The primers sequences used in the present study are listed in Table 2. Each qRT-PCR reaction was performed in duplicate. Double reference genes, elongation factor 1 alpha (EF1α) and β-actin, were used to ensure the stability of reference genes in different treatments. The relative mRNA expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.9. Statistical analysis

All values are reported as the mean ± standard error of the mean (SEM) and were tested for normality and homogeneity of variances by using Shapiro–Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) was used to test for the differences in measured parameters among the four dietary groups. When differences were obtained, specific statistical comparisons among groups were detected by using Duncan's multiple range test. Results with $P < 0.05$ values were considered statistically significant. All analyses were conducted using the Statistical Package for the Social Sciences (SPSS) version 23 (IBM, Armonk, USA).

3. Results

3.1. Growth performance and survival rate

After feeding the fish for five weeks, none of the three additives affected significantly the WG, survival rate, HSI and VSI of the treated

Table 2
Primers designed in the qRT-PCR.

TLR2	*F: GTATCTCAGTGCTCGTCGCTCA* *R: TTTCATTATCGTCTCCAGTGCG*	*XM_019360109.2*
AMPKα	*F: CTGCGTGTGAGAAGGAAGAATC* *R: CGGAAGTCAAGGAGGTAGGTT*	*KP296728*
COX2	*F: TGCTGAAAGAGGTCCACCCATACT* *R: CGCTCAGATGCTGCACGTAGTC*	*XM_003445052*
IL-10	*F: CAGCAGCAGGAGCATCAGCATT* *R: CACAGGAGGACGGTCTGAGAAGT*	*XM_005469373.3*
MIF	*F: AGCAGAAGCAGGAAGGCGAAGA* *R: CGGTACATCACCTCTGGCAACATT*	*XM_003444573.4*
Igm	*F: GCTTGACGACTGAGGATTC* *R: GTTGAAGTGGCTGTTGTGA*	*KC677037.1*
CPT1a	*F: TTTCCAGGCCTCCTTACCCA* *R: TTGTACTGCTCATTGTCCAGCAGA*	*XM_003440552*
ATGL	*F: AAAACGTCCTGGTGACCCAGT* *R: TAGGAGGAATGATGCCACAGTACA*	*XM_003440346*
TNF-α	*F: CAGAAGCACTAAAGGCGAAGAACA* *R: TTCTAGATGGATGGCTGCCTTG*	*NM_001279533*
IL-1β	*F: GAGCACAGAATTCCAGGATGAAAG* *R: TGAACTGAGGTGGTCCAGCTGT*	*XM_019365841.1*
SOD1	*F: GCCCACACTTCAATCCCTACAA* *R: GGCTCTCTTCATTTCCTCCTTT*	*XM_003446807.4*
GST	*F: TAATGGGAGAGGGAAGATGG* *R: CTCTGCGATGTAATTCAGG*	*NM_001279635.1*
Caspase-3	*F: GGAGTGGACGATACAGACGCAAA* *R: TGAAGCTGTGTGACTGGGGCTT*	*NM_001282894.1*
β-actin	*F: AGCCTTCCTTCCTTGGTATGGAAT* *R: TGTTGGCGTACAGGTCCTTACG*	*KJ126772*
EF1a	*F: ATCAAGAAGATCGGCTACAACCCT* *R: ATCCCTTGAACCAGCTCATCTTGT*	*XM_005469373.3*

fish compared to the control group (Table 3). The Nile tilapia fed on LA showed a relatively higher WG than other groups without significant difference ($P > 0.05$). The Nile tilapia fed on Gluc showed lower WG and higher survival rate than other groups, however, these changes were not significant different ($P > 0.05$). Dietary L-Ca decreased the VSI and VAI of fish ($P < 0.05$) than other groups. These results suggest that, all the three additives used did not affect growth performance and L-Ca decreased the lipid-related organ indices.

3.2. Fish body composition

The body compositions of whole fish from the four treatments are shown in Table 4. Feeding fish on Gluc diet decreased significantly crude protein compared to those fed on control and LA diets ($P < 0.05$). However, the fish fed on Gluc diet increased significantly the ash content than those fed on control diet ($P < 0.05$). Fish fed on L-CA showed lower lipid content and higher nitrogen-free extract, than those fed on the control diet ($P < 0.05$). These results indicate that, Gluc decreased protein content while L-Ca reduced lipid content.

3.3. Survival rate of the Nile tilapia after A. hydrophila challenge

Fish started to die after six days of infection and the control group showed the lowest survival rate of all the groups. After 18 days of infection, all fish in the control group died, while the survival rates in the LA, Gluc and L-Ca groups during the same period were 83%, 72% and 36%, respectively (Fig. 1). A similar survival pattern was obtained after repeating the *A. hydrophila* challenge (Supplemental materials Fig. S1). These results illustrate that, all the three additives enhanced the resistance of Nile tilapia to *A. hydrophila* infection than the control group, and the resistance abilities were higher for dietary LA and Gluc than L-Ca diet.

3.4. Influence of additives on immunity and oxidative stress enzymatic activities in serum and liver after infection

In serum, both LA and Gluc diets increased significantly the lysozyme activity compared to the control diet ($P < 0.05$), while Gluc diet also increased significantly AKP activity (Fig. 2; $P < 0.05$). Although LA, Gluc and L-Ca diets did not affect significantly the T-AOC activity of treated fish, all of them showed an increasing tendency in antioxidant enzymes activities such as SOD and CAT compared to control group. Among them, LA diet increased significantly the activities of SOD and CAT ($P < 0.05$) and Gluc diet increased significantly the CAT activity ($P < 0.05$). The level of MDA, a lipid peroxidation marker, was decreased significantly in fish fed on LA and L-CA diets compared to control diet ($P < 0.05$).

The results on the activities of immunity and antioxidant enzymes in the liver are shown in Fig. 3. Feeding fish by using LA, Gluc and L-CA diets did not affect the immune enzymes activities such as AKP and ACP. Fish fed on LA diet increased significantly the T-AOC activity in the liver compared to the other three groups. Meanwhile, the fish fed on LA, Gluc and L-CA diets increased the CAT activity compared to the control group. Accordingly, the MDA levels in the liver of fish fed on LA, Gluc and L-CA were reduced. These results suggest that, all the three additives reduce the infection-stimulated oxidative stress and LA is more efficient in improving the total antioxidant capacity than the other two additives.

3.5. Effects of the three additives on expression of genes related to disease resistance in the liver, spleen and head kidney after injection

The results on the expressions of the genes related to inflammatory response, apoptosis, antioxidant capacity and energy metabolism in the liver are given in Fig. 4. Feeding fish on LA diet did not show any effect on the expressions of the measured pro-inflammatory genes (tumor necrosis factor alpha, *TNFα* and cyclooxygenase-2, *COX2*), anti-inflammatory gene (interleukin 10, *IL-10*) and apoptosis gene (cysteine-aspartic acid protease 3, *caspase 3*). However, feeding fish on diets supplemented with Gluc and L-Ca induced the expressions of *TNFα* compared to those fed on control and LA diets ($P < 0.05$). Similarly, fish fed on Gluc diet had elevated levels of *COX2, IL-10* and *Caspase* 3 compared to those fed on control and LA diets ($P < 0.05$). Moreover, Nile tilapia fed on Gluc and L-Ca had higher expression of *IL-10* than those fed on LA and control diets, respectively ($P < 0.05$). For antioxidant genes, only the fish fed on LA had an increased expression of *SOD* and *GST* genes compared to control group ($P < 0.05$). The fish fed

Table 3
Growth performance of Nile tilapia in four groups.

	Control	α-Lipoic acid	β-Glucan	L-Carnitine
Initial body weight (g)	2.08 ± 0.04	2.01 ± 0.01	2.04 ± 0.03	2.00 ± 0.05
Final body weight (g)	6.42 ± 0.16	6.29 ± 0.07	6.16 ± 0.16	6.15 ± 0.24
Weight gain (%)	208.41 ± 5.63	212.76 ± 2.63	202.62 ± 2.66	207.25 ± 4.97
Survival (%)	94.59 ± 1.65	96.39 ± 1.81	99.12 ± 0.87	95.42 ± 3.35
HSI (%)	1.35 ± 0.07	1.53 ± 0.13	1.52 ± 0.09	1.53 ± 0.11
VSI (%)	11.15 ± 0.46	11.26 ± 0.35	11.53 ± 0.43	10.86 ± 0.39
VAI (%)	0.13 ± 0.01^{b}	0.15 ± 0.02^{b}	0.14 ± 0.02^{b}	0.08 ± 0.01^{a}

Data are expressed as mean ± SEM. Values with different superscripts within each row indicate significant differences ($P < 0.05$) among groups.

Table 4
Proximate compositions (dry matter) of whole body of Nile tilapia.

Proximate composition	Control	α-Lipoic acid	β-Glucan	L-Carnitine
Crude protein	55.85 ± 0.86[a]	56.21 ± 0.76[a]	53.41 ± 0.24[b]	54.14 ± 0.86[ab]
Lipid	18.72 ± 0.62[a]	17.12 ± 1.11[ab]	16.69 ± 1.04[ab]	15.76 ± 0.98[b]
Ash	12.42 ± 0.22[b]	12.60 ± 0.41[ab]	13.49 ± 0.30[a]	12.98 ± 0.30[ab]
Nitrogen-free extract	13.43 ± 0.83[b]	14.33 ± 1.57[ab]	16.41 ± 1.20[ab]	17.12 ± 0.75[a]

Data are expressed as mean ± SEM. Values with different superscripts within each row indicate significant differences ($P < 0.05$) among groups.

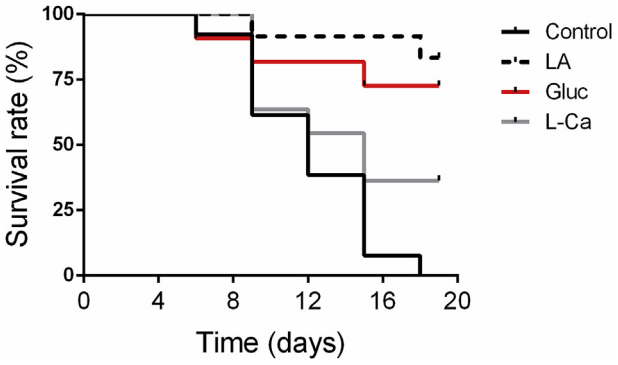

Fig. 1. Survival rate of Nile tilapia after *A. hydrophila* infection.

on LA diet had a non-significant increase in the expression of 5' adenosine monophosphate-activated protein kinase; *AMPK* gene ($P > 0.05$). The expressions of lipid catabolic genes; adipose triglyceride lipase (*ATGL*) and carnitine palmitoyltransferase 1A (*CPT1A*) were increased significantly by L-Ca compared to those fed on the other diets. Fish fed on Gluc diet only induced significantly the expression *CPT1A* compared to those fed on control diet ($P < 0.05$).

We further detected the genes involved in immunity, inflammation and apoptosis in the spleen (Fig. 5). Dietary LA increased significantly the expression of the toll like receptor (*TLR*) gene in fish compared to control and L-Ca diets ($P < 0.05$). Dietary LA also induced the specific immune gene immunoglobulin M heavy chain (*IgM*) to some extent ($P > 0.05$). Feeding fish on Gluc diet also induced significantly the expressions of *TLR* and *IgM* genes compared to those fed on control and L-Ca diets ($P < 0.05$). Nile tilapia fed on LA, Gluc and L-Ca diets decreased significantly the expression of two pro-inflammatory genes; interleukin 1 beta (*IL-1β*) and *COX2* in the spleen compared to control ($P < 0.05$). Meanwhile, only fish fed on Gluc diet increased significantly the expression of *IL-10* compared to those fed on control diet ($P < 0.05$). The expression of *caspase 3* was decreased significantly by all the three additives ($P < 0.05$), however such decrease was not statistical significant in fish fed on Gluc diet ($P > 0.05$).

We finally determined the expression of the genes related to immune responses in the head kidney (Fig. 6). Results showed that, feeding Nile tilapia on Gluc increased the expression of *TLR* gene compared to those fed on the other diets, but the change was significant only when compared to those fed on L-Ca diet ($P < 0.05$). Feeding fish on both Gluc and L-Ca diets decreased the expression of *IgM* gene compared to those fed on control diet ($P < 0.05$). Although the fish fed on Gluc increased the expression of macrophage migration inhibitory factor (*MIF*) and *IL-10* than the other three diets, it only had significant higher expression compared to LA and L-Ca diets, respectively ($P < 0.05$). The Nile tilapia fed on all the three additives decreased the expression of *caspase 3* than control group and among the three additives, those fed on L-Ca diet showed the lowest expression ($P < 0.05$). These results suggest that, all the three additives decrease the apoptosis in head kidney, while only Gluc modulated immunity and inflammation functions.

4. Discussion

In general, fish resist infectious diseases by relying on immunity function, oxidative stress reduction and energy metabolism [3,4,9]. In the present study, we found that, LA, L-Ca and Gluc protected Nile tilapia from *A. hydrophila* infection by different mechanisms. We further found that, LA, Gluc and L-Ca could all help Nile tilapia to resist *A. hydrophila* infection at different efficiencies.

The three additives used different mechanisms to protect Nile tilapia from *A. hydrophila* infection as follows. LA increased the expressions and activities of antioxidant enzymes in infected fish, suggesting LA can help to reduce oxidative stress during infection. Although LA has been previously reported to enhance the antioxidant capacity in fish under

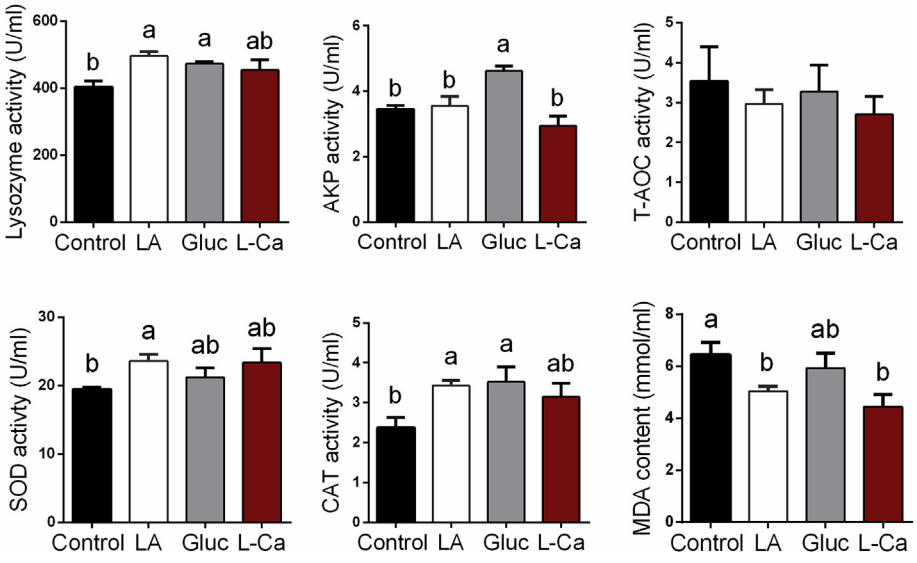

Fig. 2. The effect of the three additives on the immune enzymes (lysozyme and ACP) activities, antioxidant enzymes (T-AOC, SOD and CAT) activities and lipid peroxidation marker (MDA) content in the serum. Data are expressed as mean ± SEM (n = 6–8). Different letters above the bars show significant difference ($P < 0.05$).

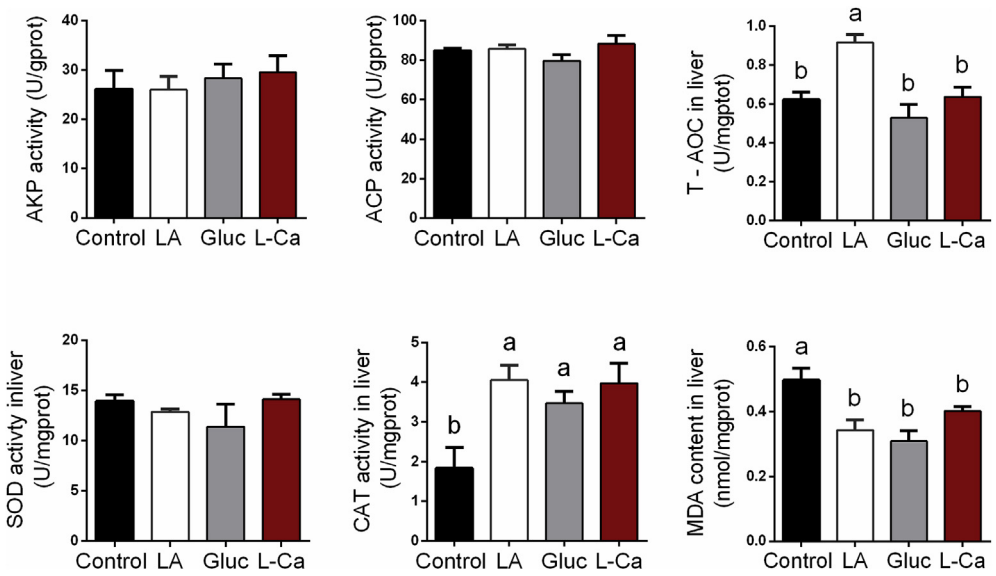

Fig. 3. The effect of the three additives on immune enzymes (AKP and ACP) activities, antioxidant enzymes (T-AOC, SOD and CAT) activities and lipid peroxidation marker (MDA) content in liver. Data are expressed as mean ± SEM (n = 6–8). Different letters above the bars show significant difference ($P < 0.05$).

metabolic or toxic stress [12,13], we mainly focused on the antioxidant function of LA under bacterial infection stress. In infected Nile tilapia, the presence of pathogen caused ROS production which induced oxidative stress. The antioxidant capacity increased in fish fed on LA diet and thus reduced the ROS accumulation to avoid excessive oxidative stress. Moreover, LA activated the lysozyme in serum, induced the expression of TLR and reduced the inflammatory response in spleen of infected Nile tilapia after five days, similar to other antioxidants such as vitamin A and vitamin E, which enhanced the immunity in mammals [37,38]. This agrees with the recently established function of LA on improving immune function and attenuate inflammatory responses in spleen and head kidney of grass carp infected by *A. hydrophila* for 14 days [15]. Lysozyme is an important part of the innate immunity function for cleaning pathogen and TLR is an important pathogenic receptor, which modulate both the innate and specific immunity [39], suggesting LA can activate the immune function through stimulation of the TLR. These results demonstrate that, LA help Nile tilapia to resist *A. hydrophila* infection mainly by improving the antioxidant capacity and

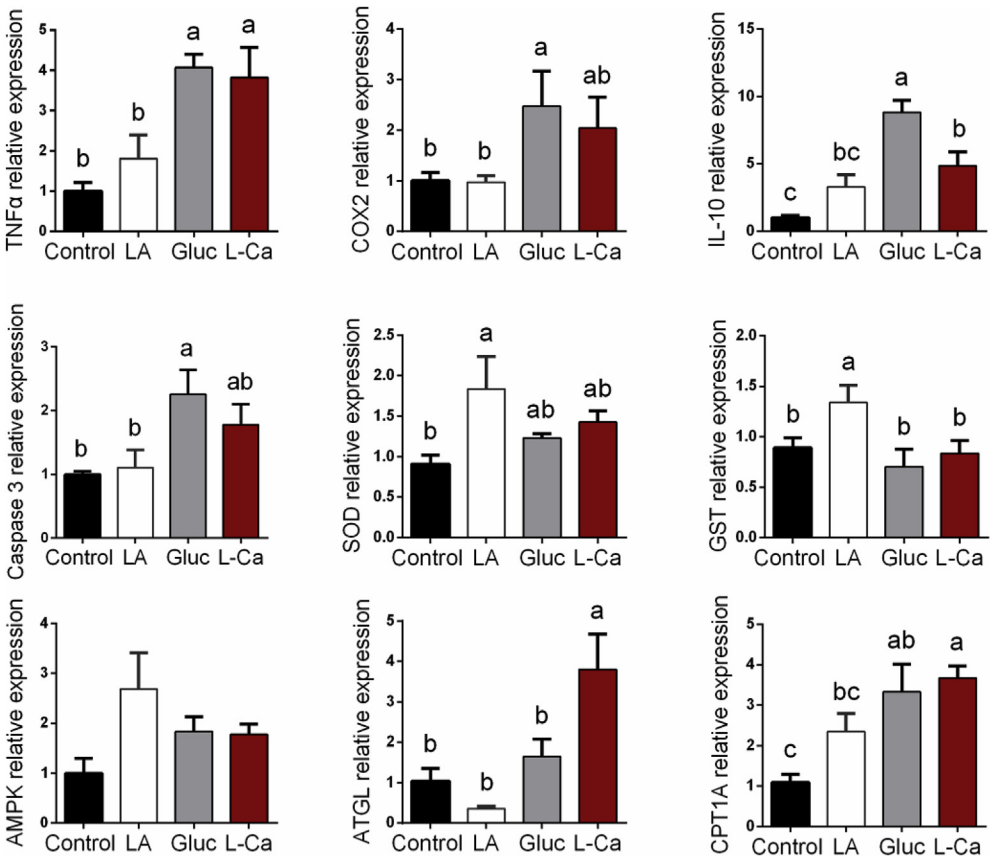

Fig. 4. Expression of genes function for pro-inflammation (TNFα and COX2), anti-inflammation (*IL-10*), apoptosis (*Caspase 3*), anti-oxidation (*SOD* and *GST*), energy metabolism (*AMPK*) and lipid catabolism (*ATGL* and *CPT1A*) in liver. Data are expressed as mean ± SEM (n = 5–6). Different letters above the bars show significant difference ($P < 0.05$).

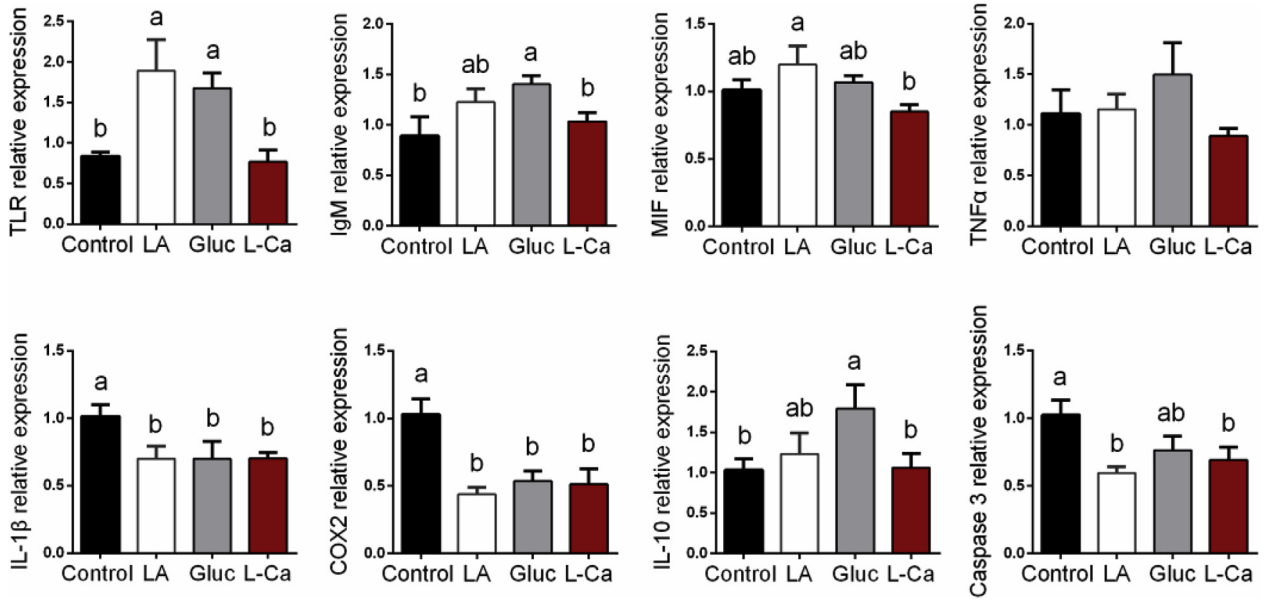

Fig. 5. Expression of genes function for immune function (*TLR* and *IgM*), pro-inflammation (*MIF, TNFα, IL-1β* and *COX2*), anti-inflammation (*IL-10*) and apoptosis (*Caspase 3*) in spleen. Data are expressed as mean ± SEM (n = 5–6). Different letters above the bars show significant difference ($P < 0.05$).

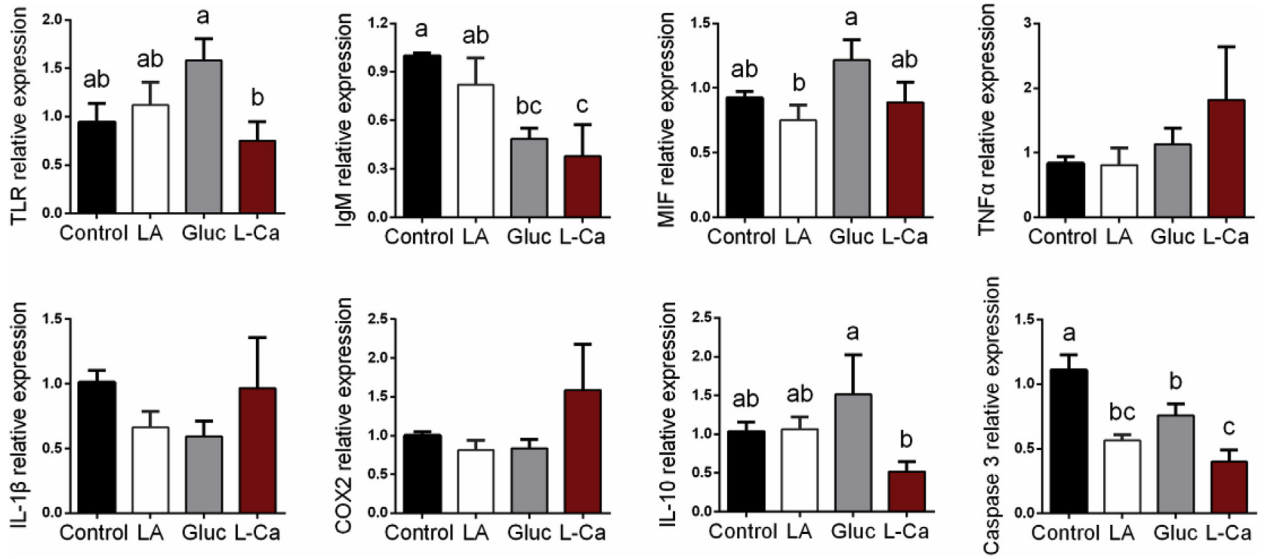

Fig. 6. Expression of genes related to immune function (*TLR* and *IgM*), pro-inflammation (*MIF*, *TNFα*, *IL-1β* and *COX2*), anti-inflammation (*IL-10*) and apoptosis (*Caspase 3*) in the head kidney. Data are expressed as mean ± SEM (n = 5–6). Different letters above the bars show significant difference ($P < 0.05$).

immune response.

Inflammation is an important part of innate immunity [40]. Previous studies have reported that Gluc enhances the immunity function by stimulating inflammatory response [18,41]. In this study, Gluc increased the lysozyme and AKP activities in serum and enhanced the expression of *TLR* and *IgM* in spleen, consistent with previous researches in Nile tilapia [16] and other fishes [2]. Gluc showed different expression patterns of pro-inflammation factors in different organs; increased the *TNFα* and *COX2* in liver, while decreased the *IL-1β* and *COX2* in spleen as reported previously in different organs in common carp [41]. It also increased the anti-inflammation factor; *IL-10* in liver, spleen and head kidney tissues. The increase in anti-inflammation response in Nile tilapia due to dietary Gluc indicated its ability to induce inflammatory modulation. The induced inflammation in Nile tilapia after dietary Gluc was not only intended for cleaning pathogen, but also signifies tissue damage when fish were under stress. The reduced inflammation response indicated a protective effect of Gluc in spleen of infected fish as reported previously [15]. We further found Gluc increased the activity of CAT and decreased the infection-caused oxidative stress in tissues of infected Nile tilapia, similar to results reported in grass carp [22] and rainbow trout [19]. These results show that, Gluc increased the antioxidant capacity of fish to resist pathogen during infection. In liver, Gluc also increased the expression of *CPT1A*, suggesting Gluc improved fatty acid catabolism similar to results obtained in mammals, in which Gluc provided beneficial effects by decreasing the high fat diet-induced lipid accumulation and glucose intolerance [42]. However, research in turbot showed Gluc had no effect on lipid catabolism [43]. The increase in lipid catabolism in our study may be attributed to higher energy demand caused by the Gluc immune activation. In short, Gluc helps Nile tilapia to resist infectious disease by enhancing inflammatory response, antioxidant capacity and lipid catabolism.

L-Ca have been reported to act as an immunostimulant in turbot [44], while in the present study, L-Ca neither affected the immune

enzyme activity of Nile tilapia in serum nor changed the expression of immune genes in spleen and head kidney. These results suggest that, L-Ca did not enhance immune function in infected Nile tilapia. Dietary L-Ca induced similar effects on pro-inflammatory factors to those obtained on Gluc diet in the liver and spleen of Nile tilapia. These results demonstrate that, L-Ca can also activate inflammation response in the liver and reduce the damages in spleen. However, L-Ca showed no effect on anti-inflammatory factor; *IL-10*, suggesting the inflammatory modulation capacity of L-Ca was not as powerful as that of Gluc diet. Interestingly, L-Ca also increased the activity of CAT in the liver and reduced the oxidative stress both in serum and liver. The anti-oxidative function of L-Ca have been reported in some aquatic animals such as crayfish (*Astacus leptodactylus leptodactylus*) [45] and common carp [46]. In tilapia, L-Ca performed anti-oxidative function only when fish were under oxidative stress status [32], which is consistent with our result in infected fish. The results of our study further demonstrated that, L-Ca decreased the lipid content of whole fish after diet treatment and increased the expressions of lipolysis genes; *ATGL* and mitochondrial fatty acid β-oxidation; *CPT1A* in liver during infection, consistent to its ability on improving lipid utilization in many fishes [26,46]. We mainly tested the lipid catabolism to compare the energy metabolism of additives because LA and L-Ca have been reported to regulate lipid metabolism in other animals [23,42,47]. These results suggest that, L-Ca largely improved the lipid catabolism through enhancing the mitochondrial fatty acid β-oxidation. The β-oxidation is an important part of energy supply, subsequently blocking mitochondrial fatty acid β-oxidation have been found to reduce resistance of Nile tilapia to *A. hydrophila* infection [9]. Altogether, L-Ca can help to resist *A. hydrophila* infection in Nile tilapia through enhancing antioxidant capacity and lipid utilization.

Our results showed that, all the three additives studied exerted extensive functions on immunity, anti-oxidation and energy metabolism at different efficiencies. We compared the effects of additives on survival, tissues damages, immune functions, antioxidant capacity and energy metabolism (Fig. 7). All the additives used in our study decreased the tissue damages caused by *A. hydrophila* infection in Nile tilapia, but at different efficiencies. Although the effects of LA on fish immunity have been discussed in grass carp [15], its ability to resist pathogen infection in fish had not been reported before. Unexpectedly, we found that, the dietary LA ability to resist infection caused by *A. hydrophila* in Nile tilapia was similar to that of Gluc diet, but higher than that of L-Ca diet. Our results showed that, although all the three additives used increased the activities of antioxidant enzymes, Gluc and L-Ca diets had lower antioxidant capacity than LA diet. Moreover, dietary LA induced partly the innate immunity while L-Ca showed no effect. In fact, Gluc induced higher level of immune function than the other two additives. Among the three additives, L-Ca induced the highest utilization of lipid to supply energy and Gluc induced fatty acid β-oxidation at a lower level than L-Ca, while LA showed no effect on lipid catabolism. Based on the specific benefits of the three additives, we hypothesize that their combined application could elicit synergistic effects and better infection resistance than using individual additive. Moreover, based on multi-function nature of the additives used, their combined application may reduce the amount needed because of the synergistic effects. However, these hypotheses need further studies to confirm them.

5. Conclusion

Taken together, the dietary LA, L-Ca and Gluc used in our study helped Nile tilapia to resist bacterial infection with different mechanisms and efficiencies. Apparently, dietary LA induced high infection resistance and efficiency through antioxidant function and by increasing slightly the immune function. On the other hand, dietary Gluc induced strong bacterial infection resistance to Nile tilapia by stimulating high efficiency immune function and enhancing slightly the antioxidant capacity and lipid catabolism. Lastly, dietary L-Ca increased slightly the infection resistance of Nile tilapia to *A. hydrophila* through high lipid catabolism efficiency and slightly increasing the antioxidant capacity. In general, all these mechanisms used by dietary LA, Gluc and L-Ca provide useful information for protecting fish in aquaculture for sustainable production.

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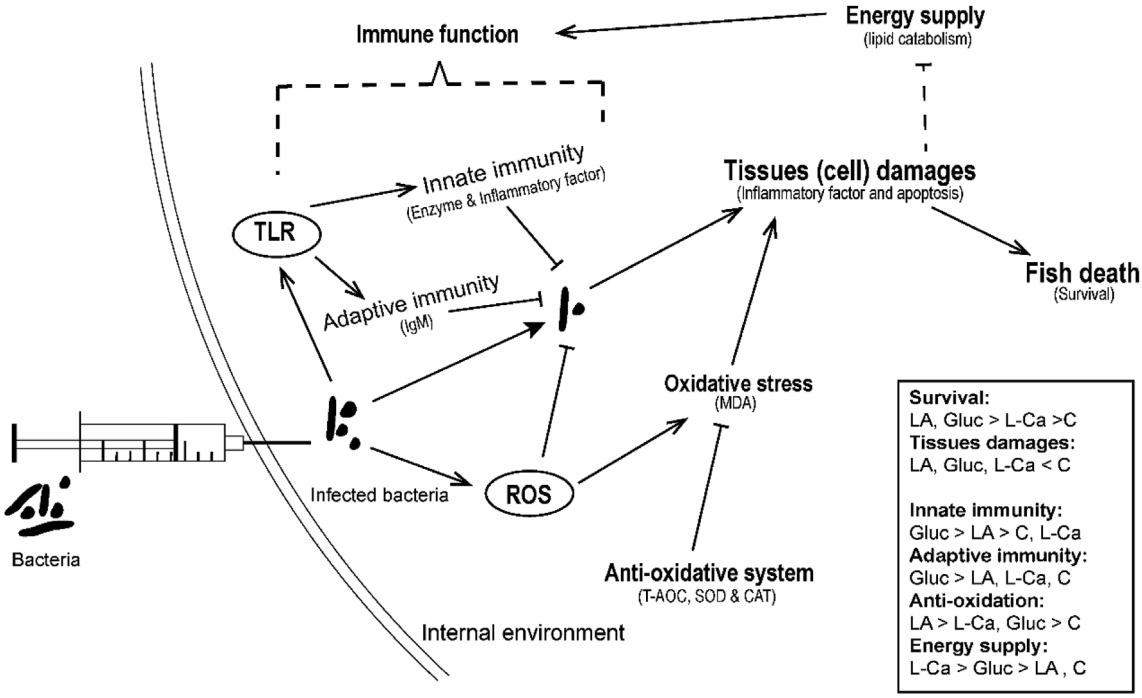

Fig. 7. Summary of the protective mechanisms of the additives in resisting *A. hydrophila* infection in Nile tilapia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.023.

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